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APPLICATION FOR LETTERS PATENT

for

**AN IMPROVED PEPTIDE, IMMUNOGENIC COMPOSITION AND VACCINE OR
MEDICAL PREPARATION, A METHOD TO IMMUNISE ANIMALS AGAINST THE
HORMONE LHRH, AND ANALOGS OF THE LHRH TANDEM REPEAT PEPTIDE
AND THEIR USE AS VACCINE**

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Addendum

1. AN IMPROVED PEPTIDE, IMMUNOGENIC COMPOSITION AND VACCINE OR MEDICAL PREPARATION, A METHOD TO IMMUNISE ANIMALS AGAINST THE HORMONE LHRH, AND ANALOGS OF THE LHRH TANDEM REPEAT PEPTIDE AND THEIR USE AS VACCINE

095593-05420

**AN IMPROVED PEPTIDE, IMMUNOGENIC COMPOSITION AND VACCINE
OR MEDICAL PREPARATION, A METHOD TO IMMUNISE ANIMALS
AGAINST THE HORMONE LHRH, AND ANALOGS OF THE LHRH TANDEM
REPEAT PEPTIDE AND THEIR USE AS VACCINE**

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This application is a continuation-in-part of application serial number 09/274,048 filed 22 March, 1999, which is a continuation of application serial number 08/981,557 filed 7 June 1995 as a national entry application under PCT/NL96/00223 filed 6 June 1996, which claims priority under U.S. application 08/447,298 filed 7 June 1995 and U.S. application 08/476,013 filed 7 June 1995.

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BACKGROUND OF THE INVENTION

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Field of the Invention: This invention relates to a peptide suitable for eliciting an immune response against forms of Gonadotropine Releasing Hormone (GnRH) also referred to as Luteinizing Hormone Releasing Hormone (LHRH). The invention further relates to immunogenic compositions and vaccines, pharmaceuticals, and other medicinal preparations based on such a peptide. The invention further relates to the use of such a vaccine or medicinal preparation in a method of immunizing a mammal against GnRH to influence reproductive or behavioral characteristics of that mammal and in a method of improving the carcass quality of pigs. The invention also relates to a peptide suitable for eliciting a selective immunogenic response against GnRH-I or GnRH-II. Further the invention relates to antibodies against GnRH-I, to compositions comprising these antibodies and to the use of the peptides in pharmaceutical compositions or in the preparation for a medicament for the treatment of prostate cancer.

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State of the Art: GnRH-I (in the literature generally depicted as GnRH) is a small 10 amino acid long peptide (decapeptide) from the hypothalamus. The amino acid sequence of GnRH-I (SEQ ID NO: 1) can be represented by the following three-letter code:



RMO
4/15/03

1997-1998		1998-1999		1999-2000		2000-2001		2001-2002		2002-2003		2003-2004		2004-2005		2005-2006		2006-2007		2007-2008		2008-2009		2009-2010		2010-2011		2011-2012		2012-2013		2013-2014		2014-2015		2015-2016		2016-2017		2017-2018		2018-2019		2019-2020		2020-2021		2021-2022		2022-2023		2023-2024		2024-2025		2025-2026		2026-2027		2027-2028		2028-2029		2029-2030		2030-2031		2031-2032		2032-2033		2033-2034		2034-2035		2035-2036		2036-2037		2037-2038		2038-2039		2039-2040		2040-2041		2041-2042		2042-2043		2043-2044		2044-2045		2045-2046		2046-2047		2047-2048		2048-2049		2049-2050		2050-2051		2051-2052		2052-2053		2053-2054		2054-2055		2055-2056		2056-2057		2057-2058		2058-2059		2059-2060		2060-2061		2061-2062		2062-2063		2063-2064		2064-2065		2065-2066		2066-2067		2067-2068		2068-2069		2069-2070		2070-2071		2071-2072		2072-2073		2073-2074		2074-2075		2075-2076		2076-2077		2077-2078		2078-2079		2079-2080		2080-2081		2081-2082		2082-2083		2083-2084		2084-2085		2085-2086		2086-2087		2087-2088		2088-2089		2089-2090		2090-2091		2091-2092		2092-2093		2093-2094		2094-2095		2095-2096		2096-2097		2097-2098		2098-2099		2099-2100		2100-2101		2101-2102		2102-2103		2103-2104		2104-2105		2105-2106		2106-2107		2107-2108		2108-2109		2109-2110		2110-2111		2111-2112		2112-2113		2113-2114		2114-2115		2115-2116		2116-2117		2117-2118		2118-2119		2119-2120		2120-2121		2121-2122		2122-2123		2123-2124		2124-2125		2125-2126		2126-2127		2127-2128		2128-2129		2129-2130		2130-2131		2131-2132		2132-2133		2133-2134		2134-2135		2135-2136		2136-2137		2137-2138		2138-2139		2139-2140		2140-2141		2141-2142		2142-2143		2143-2144		2144-2145		2145-2146		2146-2147		2147-2148		2148-2149		2149-2150		2150-2151		2151-2152		2152-2153		2153-2154		2154-2155		2155-2156		2156-2157		2157-2158		2158-2159		2159-2160		2160-2161		2161-2162		2162-2163		2163-2164		2164-2165		2165-2166		2166-2167		2167-2168		2168-2169		2169-2170		2170-2171		2171-2172		2172-2173		2173-2174		2174-2175		2175-2176		2176-2177		2177-2178		2178-2179		2179-2180		2180-2181		2181-2182		2182-2183		2183-2184		2184-2185		2185-2186		2186-2187		2187-2188		2188-2189		2189-2190		2190-2191		2191-2192		2192-2193		2193-2194		2194-2195		2195-2196		2196-2197		2197-2198		2198-2199		2199-2200		2200-2201		2201-2202		2202-2203		2203-2204		2204-2205		2205-2206		2206-2207		2207-2208		2208-2209		2209-2210		2210-2211		2211-2212		2212-2213		2213-2214		2214-2215		2215-2216		2216-2217		2217-2218		2218-2219		2219-2220		2220-2221		2221-2222		2222-2223		2223-2224	
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ovary, testis, thymus, placenta and hypothalamus of the rat (Oikawa et al.,
Endocrinology, 1990, 127, 2350-2356). Expression of GnRH was detected in immune
tissue (spleen, thymus and lymphocytes) of pigs (Weesner et al., Life Sci, 1997, 61,
1643-1649).

5 GnRH-II is expressed in many tissues outside the brain, and is found in especially
high concentrations in the kidneys, bone marrow and prostate. The presence of GnRH-II
in diverse tissues other than the brain suggests that GnRH-II may have multiple
functions. In addition, the strictly conserved structure of the GnRH-II peptide throughout
diverse vertebrate species suggests that this neuropeptide possesses vital bioactivities.
10 Until now, however, the functions of GnRH-II have been practically unknown. Several
types of differentiated lymphocytes, such as T- and B-lymphocytes and mast cells,
produce GnRH and GnRH-like peptides. Significant numbers of the latter cell type are
present in kidney, bone marrow and prostate, perhaps contributing to the high GnRH-II
expression in these tissues. GnRH II seems less involved in reproduction as compared to
15 GnRH-I. In the hypogonadal mouse, mouse which lack the GnRH-I gene, GnRH-II
producing cells are present in the same distribution as in normal mouse, but this is not
sufficient to cause normal gonadal development in these mouse (Chen et al. FEBS Letters
435 (1998) 199-203). However macaques in luteal phase of the menstrual cycle showed
a marked increase in plasma luteinizing hormone concentrations after intravenous
20 administration of GnRH-II, but this increase could not be induced during the mid
follicular phase (Lescheid et al. Endocrinol. 138 (1997) 5618-5629.)

BRIEF SUMMARY OF THE INVENTION

25 The invention now provides the insight that by providing peptide sequences that
allow discrimination between the different types of GnRH, more adequate and efficient
use can be made of the variation or difference in immunological response to the different
types of GnRH. More particularly, the invention provides the insight that improvements
in the efficacy and selectivity of the vaccines against GnRH-I can be achieved.

30 Immunization against GnRH-I is effective in neutralizing GnRH-I and results in
reduced gonadotropin levels and blocking of gonadal steroid synthesis. However,

nothing is known about any physiological effects of the antibodies raised against GnRH-I on the function of GnRH-II. As GnRH-II is mainly synthesized and secreted in the kidneys antibodies raised against GnRH-I that cross-react with GnRH-II may affect kidney function. To obviate possible side effects of GnRH-I immunization on kidney function it would be desirable to direct the antigenic response of an immunocastration vaccine specifically toward GnRH-I and to avoid possible harmful side-effects due to neutralization of non-gonadal GnRH-II.

If the reproductive capacity alone, often with its accompanying sexual behavior, of a species needs to be annulled, it would be preferred to aim at an immunocastration vaccine specifically neutralizing GnRH-I. Hence the desire to come to a selective immunization against Gonadotropine Releasing Hormone(s), preferably selective against GnRH-I.

In veterinary medicine, 100% effective immunization against GnRH-I could be used for the sterilization of, e.g., small domestic animals such as male and female cats and dogs, or for the treatment of aggressiveness in male dogs and bulls, simply by vaccination instead of by drastic surgery such as castration or ovariectomy. Other conceivable reasons for immunization against GnRH-I are to prevent heat in female animals, such as dogs, cats and cows, and restlessness in male animals being fattened for slaughter.

In human health care, immunization against GnRH-I and/or GnRH-II can be used in the treatment of prostate cancer and breast cancer and, if required, in the treatment of some forms of pituitary carcinoma. In the case of prostate cancer it might be more desirable to neutralize both GnRH-I and GnRH-II, as the latter isoform is also highly expressed in prostate tissue.

Another use of a vaccine against GnRH-I is in the field of stock breeding, particularly the fattening of pigs for slaughter. The meat of male, sexually mature pigs (boars) has a typical odor, the so-called boar taint or boar odor. In the testes of the sexually mature pig, many C19-delta-16 steroids are formed which are stored in the fat tissue of the animal (Patterson, *J. Sci. Food Agric.* 19, 31-38 (1968); Brooks en Pearson, *J. Anim. Sci.* 62, 632-645 (1986); Claus, *Zeitschrift. Tierzüchtg. Züchtungsbiol.* 93,

38-47 (1976); Claus, *Acta Endocrinol. (Copenh.)* 91, Suppl. 225, 432-433 (1979)). These steroids are mainly responsible for the formation of the disagreeable urine-like odor when the meat is heated (Fuchs, *Swedish J. Agric. Res.* 1, 233-237 (1971); Bonneau, *Livest. Prod. Sci.* 2, 687-705 (1982)). Owing to this unpleasant odor, meat of male sexually mature pigs is generally unsuitable for consumption and unfit for export. Because about 10% of the male slaughter pigs are already sexually mature before the slaughter time, this potentially entails a great loss for the pig farming industry.

In order to control and prevent these losses, nearly all male piglets are castrated when they are young, with a surgical procedure that is generally executed without any form of anaesthesia. Apart from the animal unfriendly aspect of such a castration, castration also leads to infections, growth inhibition, and a final carcass quality inferior to that of an intact animal, at least as long as that intact animal has not yet developed boar taint (Walstra, *Livest. Prod. Sci.* 1, 187-96 (1974)).

An animal friendly alternative consists in the reduction of the GnRH-I concentration in the pig pituitary by means of immunization against GnRH-I, the so-called immunocastration. This reduction in GnRH-I levels leads to a reduction in the concentrations of biologically active FSH and LH, which in turn will inhibit development of the testes in the growing animals and inhibit the synthesis of testicular steroids, including androstenone, testosterone and estrogens. This method prevents the occurrence of boar taint in male pigs at slaughter time and makes surgical castration unnecessary as androstenone levels are reduced to levels low or undetectable (Oonk et al., 1995, *Livestock production Science* 42, 63-71).

A strict requirement for an acceptable vaccine against boar taint is that in almost all pigs development of the testes is delayed to such an extent that boar taint will not have occurred at the time of slaughter, and that in the case that the vaccine does not reduce testis development in an animal, this can be easily detected in a too large testis size in comparison to successfully immunocastrated pigs.

In the existing literature and previous patent applications regarding the anti-fertility properties of vaccines against GnRH-I, the results of vaccinations often appear to be variable. In most of the described studies, either a small percentage of the vaccinated

animals do not respond to the vaccination, or large doses of vaccine, multiple vaccinations or commercially unacceptable adjuvants are needed to produce the desired effect (Hoskinson et al., 1990, Austr. J. Biotech. 4, 166-170; Falvo et al., (1986) J. Anim. Sci. 63:986-994; Clarke et al., 1998, Endocrinology 139, 2007-2014; Adams T.E. and B.M. Adams, Feedlot performance of steers and bulls active immunized against Gonadotropine-Releasing Hormone, J. Anim. Sci. 1992, 70: 1691-1698; Brown et al., Immunization of sheep against GnRH-I early in life: effects of reproductive function and hormones in rams, Journal of reproduction and Fertility (1994) 101, 15-21; Ferro et al., Immunological castration using a Gonadotropine-releasing Hormone analogue conjugated to PPD, Food and agricultural immunology, 1995, 7, 259-272; U.S. patent 4,608,251; Int. patent appl. WO 88/05308).

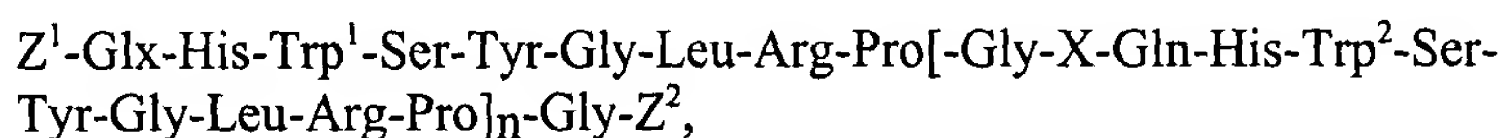
Some studies suggested an efficacy of 100% of a vaccine against GnRH-I, but the vaccine was not tested in a large number of animals (Ladd et al. (1994), Development of an antifertility vaccine for pets based on active immunization against Luteinizing Hormone releasing hormone, Biology of Reproduction 51, 1076-1083 ; J.G. Manns and S.R. Robbins (1997). Prevention of boar taint with a recombinant based GnRH vaccine, In: Boar taint in entire male pigs, Proceedings of a meeting of the EAAP working group "Production and Utilisation of Meat from Entire Male Pigs", EAAP Publication No. 92, 137-140;); other studies report the efficacy of the vaccine as the mean value of the treated animals, since individual values did not show a clear difference between immunized and untreated controls (Bonneau et al., J. Anim. Sci. 72, 14-20 (1994); Hennesy et al., 1997. Elimination of boar taint: a commercial boar taint vaccine for male pigs. In: Bonneau, M., Lundström, K. and Malmfors, B. (Eds.), Boar taint in entire male pigs. Wageningen Pers, Wageningen, EAAP Publication No. 92., 141-145).

The difficulty in preparing this type of vaccines probably is caused by the phenomenon of tolerance. Self substances such as hormones are not recognised, as foreign but rather are tolerated by the immune system. Normally no antibodies are elicited against self substances. In order for a vaccine to be successful, it must be sufficiently foreign. Only when the vaccine is foreign enough will the immune system not tolerate the vaccine and the production of antibodies be induced. Conversely,

however, the antibodies must still be capable of recognizing the hormone, and thus the vaccine cannot be too 'foreign'.

As these conditions appear to be mutually exclusive, it was not certain, until recently, if such substances could be prepared at all. One attempt to produce GnRH-like peptide vaccines consisted of the replacement of Gly at position 6 of the GnRH-I decapeptide by a dextrorotary amino acid (D-Tryp; Chaffaux et al., *Recueil de Médecine Vétérinaire* 161 (2), 133-145, 1985). It was, however, demonstrated that a vaccine preparation containing this modified GnRH-peptide performed even less well than the normal GnRH-I decapeptide (European Patent application-464,124 A).

Recently, we have shown definitively that it is possible to elicit an effective antibody response in all individuals vaccinated against GnRH-I (Meloan et al., *Vaccine* 12, 741-746 (1994)). In these experiments pigs were vaccinated twice with an GnRH-I vaccine that departs from the classical type of GnRH-I vaccine (GnRH-I coupled to a carrier protein, in Freund's adjuvant), namely the tandem-GnRH-I vaccine (European patent nr. 0464124). In this publication a peptide is described which is characterized in that it comprises at least 2 GnRH-I sequences in tandem (SEQ ID NO: 3) according to the general formula



in which amino acids are designated according to the three-letter code, Trp¹ and Trp² are tryptophan (Trp) or formylated tryptophan (N(indole)-formyl-tryptophan), n is a number having a value of at least 1, X is either a direct bond or a spacer group between the amino acids Gly and Gln, Z¹-Glx is either pGlu (pyroglutamic acid) or Gln having attached thereto a tail comprising one or more additional amino acids, and Gly-Z² is either Gly-NH₂ or Gly having attached thereto a tail comprising one or more additional amino acids. In this general formula, X may be a direct bond between the amino acids glycine and glutamine, i.e. these amino acids are interconnected directly without an intermediate link (via the normal peptide bond). The tandem-GnRH-I vaccine invention also comprises peptides in which the GnRH-I sequences are interconnected via spacers. The nature of the spacer group may vary greatly, from one or more amino acids to a shorter or

longer hydrocarbon chain and other compound groups or molecules. In the above general formula, Z¹-Glx preferably stands for pGlu (pyroglutamic acid), but can also stand for Gln having attached thereto a tail comprising one or more additional amino acids, e.g., to be used for coupling of the peptide to a carrier protein. In the above
5 general formula, Gly-Z² stands for, e.g., Gly-NH₂, or Gly having attached thereto a tail comprising one or more additional amino acids, e.g., to be used for coupling of the peptide to a carrier protein. Preferably, Gly-Z² stands for Gly-Cys-NH₂, the C terminal cysteine being added in connection with a possible coupling of the peptide to a carrier protein.

10 From WO 96/40755 it is known that the tandem-dimer principle applied to a variant of the GnRH-I molecule resulted in a vaccine that was highly effective in several mild adjuvants, namely Specol and a double oil emulsion, and was also effective in low doses. In this case, the variant of the GnRH-I molecule was formed by substitution of the sixth amino acid Gly of the decapeptide by a dextrorotatory (D-) amino acid, D-Lys,
15 following which the resulting peptide was dimerised and coupled to a common carrier compound, ovalbumine. Thus, whereas a vaccine using D-amino acid substitutions of Gly at position 6 of the original and a single GnRH-I decapeptide with a D-amino acid decreased the immunogenicity as compared to the original GnRH-I sequence (Chaffaux et al., Recueil de Médecine Vétérinaire 161 (2), 133-145, 1985), such substitutions with a
20 D-amino acid applied to a tandem-dimer GnRH-I vaccine were able to generate even more immunogenic GnRH-I vaccine preparations. Nevertheless, the method for vaccination required a repeat dosage of the vaccine in order to be completely effective. The necessity of an additional booster dosage in order to achieve essentially 100% vaccination of mammals against GnRH-I is a disadvantage of the known peptides. We
25 also found that in certain cases use of the (D-Lys⁶)GnRH I tandem dimer (i.e. the GnRH I tandem dimer, with and without the D-Lys⁶ replacement) resulted in very low but still measurable amounts of testosterone, which is undesirable and a disadvantage of the (D-Lys⁶)GnRH I tandem dimer.

The present invention provides peptide sequences and that provide alternatives to the tandem D-Lys⁶ GnRH-I when applied in vaccines that result in vaccines that are effective for immunocastration.

5 An aspect of the present invention is the determination of the extent to which the amino acids in the tandem GnRH-I sequence can be varied while the resulting substituted tandem GnRH-I is still able to produce an immunogenic response to GnRH-I sufficient for immunocastration. Thus, the invention provides for the generation of a peptide sequence that can induce the production of antibodies against GnRH-I, which are also sufficiently competitive, both in amount and activity.

10 A further aspect of the present invention is to provide to a peptide sequence that selectively induces the production of antibodies against GnRH-I, while inducing little or no immune response towards GnRH-II. A preferred embodiment thereof is a peptide sequence that not only selectively induces the production of antibodies against GnRH-I but is also effective in immunocastration, while an immune response to GnRH-II is
15 reduced or absent.

The present inventors have found that in the tandem GnRH-I peptide sequence various amino acids can be replaced, resulting in a decreasing resemblance to the self-hormone while at the same time retaining or even increasing the ability of the peptide to elicit GnRH-I binding antibodies. Also, certain replacements of amino acids in the
20 GnRH-I peptide sequence result in a selective immune response towards GnRH-I and to a reduced or absent immuneresponse towards GnRH-II.

In an aspect of the invention, certain modified tandem GnRH-I peptide sequences provide for vaccines that are not only capable of reducing testes growth in male animals but are also capable of essentially reducing testosterone levels to a degree that they can
25 not be determined by conventional techniques.

Further, vaccines prepared from these peptide-sequences express an activity that in most cases eliminated the need for the second booster immunization, as with the conventional D-Lys⁶ tandem GnRH-I, to achieve essentially 100% activity. An activity or efficacy of 100 % in the terms of the present invention is defined as a testosterone

level that is essentially undetectable with conventional techniques after a single vaccination.

One of the most notable features of the present invention is that antibodies raised by these alternative GnRH vaccines discriminate between GnRH-I and GnRH-II. Thus peptides according to the invention express an increased or retained activity against GnRH-I, while at the same time a reduced or absent immune response to GnRH-II is found. This allows for the development of peptides that express an inverse effect, that is, they express an increased or retained activity against GnRH-II while at the same time expressing a reduced or absent immune response against GnRH-I.

The invention relates in one aspect to a peptide that comprises a modified tandem GnRH-I decapeptide sequence whereby vaccination with the peptide in a suitable dosage allows for a testosterone level that is essentially non-detectable.

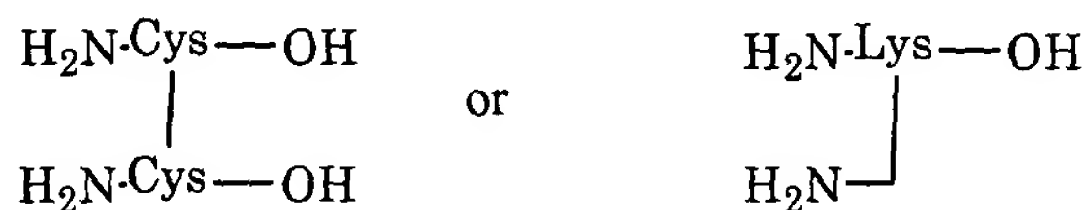
The invention also relates to a peptide that comprises at least two coupled GnRH-I decapeptide sequences, optionally coupled through a spacer, which allows for an immunogenic response that allows for the effective discrimination between GnRH-I and GnRH-II.

The peptides according to the invention are sufficiently like the hormone but at the same more 'foreign' to the immune system and have an increased capability to induce the production of antibodies directed against the hormone.

A feature of the invention is that individual tandem units can be dimerised to further enhance its immunogenicity without losing the possibility of coupling the peptide or peptide composition to a carrier compound protein.

The techniques for dimerisation and coupling of the tandem to a carrier similar to those described in WO 96/40755 may be used. It is also envisioned that peptides containing only a portion of the GnRH-I or II peptide sequences can be used in the present invention. Examples thereof are nonapeptides and undcapeptides.

The linkers for use in the peptides according to the invention can be selected from the linkers described elsewhere in this application or linkers such as



Linkers are used for coupling two or more dimerised peptide sequences. The amino acid which is used to replace the amino acid in the tandem peptide sequences is preferably an amino acid which is a relative simple one, such as alanine. In a preferred embodiment therefor the different amino acid is alanine. Other amino acids can also be used to replace the amino acid in the tandem decapeptide sequence. Preferably only conservative replacements are carried out. Conservative replacements are amino acid substitutions in which bulky amino acids are replaced by bulky amino acids, aromatic amino acids by aromatic amino acids etc. These concepts are well known to those skilled in the art.

Spacers can be placed between the peptides according to the invention. This allows for the formation of multimers. Suitable spacers are known in the art.

DESCRIPTION OF THE DRAWINGS

Figure 1: Competition for binding of iodinated GnRH with antiserum by GnRH (open circles), GnRH-II (solid circles), control peptide (solid squares) and no peptide (asterix). Serum is diluted 1/10000 and increasing peptide concentrations (0.25, 2.5 25 pmol) per well were added. In graphs A-C sera with increasing binding capacity for GnRH-II are shown. Horizontal axis: amount of peptide (pmol per well); vertical axis: Binding capacity (counts per minute).

Figure 2: Percentage of maximum binding of antiserum to iodinated GnRH after competition with GnRH-I (dark bars) or GnRH-II (light bars) for sera obtained after immunization with the peptides indicated in the left top corner of each graph. Antiserum obtained 3 weeks after boosterimmunisation was diluted 1:10000 and peptides for

competition were added in 250 nmol per liter. Horizontal axis : animal identification #; Vertical axis: percentage of maximum binding.

Figure 3 Percentage of maximum binding of antiserum to iodinated GnRH after competition with GnRH-I (dark bars) or GnRH-II (light bars) for antiserum against pE1A collected at 7 wpv (A), and at 10 wpv (B) and for antiserum against S4A collected at 7 wpv (C), and at 10 wpv (D). Antiserum obtained at 7 wpv and at 10 wpv was tested in an 1/2000 and 1/10000 dilution respectively. 25 pmol peptide for competition was added per well. Horizontal axis: animal identification #; Vertical axis: percentage of maximum binding.

Figure 4: Immunocastration efficacy score of several groups of pigs immunized with GnRH-tandem-dimer peptides (62 mg) conjugated to ovalbumine and Specol used as an adjuvant. The starting peptide was GnRH tandem (Cys-OH) dimer (abbreviated Cys-OH). Of this peptide all alanine scan peptides were used for immunization. Immunocastration efficacy was rated on a scale of 1 to 4 (1= immunocastration did not occur, 2 = a minority of the pigs was immunocastrated, 3 = a majority of the pigs was immunocastrated, 4 all pigs were immunocastrated).

DETAILED DESCRIPTION OF THE INVENTION

In a feature of the invention, the peptides according to the invention have the general formula given by (SEQ ID NO: 4), or by the following one-letter code:

pEHWSYKLRPGQHWSYKLRPGC#

In this general formula Q stands for Gln and may be preceded by X where X stands for a spacer. Several of these amino acids have been replaced with other amino acids. In the formulas the position of the substitution is depicted **bold** and underscored. Capital letters stand for Levorotatory amino acids, lower case letters stand for Dextrorotatory amino acids, e.g. K: L-Lys; k:D-Lys. Subsequently their immunogenic response has been determined when coupled to a carrier, generally ovalbumine, but other carriers such as KLH, BSA can be used.

It will be clear that the sequence of the GnRH-II peptide (SEQ ID NO: 2) determines which of the amino acids can be replaced in such manner that an effective

discrimination based on the immune response is still possible between the two sequences GnRH-I, and GnRH-II.

To determine whether antibodies raised by the GnRH vaccines according to the present invention discriminated between GnRH-I and GnRH-II, a GnRH antibody binding assay was performed in order to determine whether antibodies raised against GnRH-I-tandem dimer peptide or its alanine replacement analogs bind to GnRH-II or lack binding to GnRH-II. Serum dilutions were preincubated with either GnRH-I, GnRH-II, a control peptide or no peptide. Next iodinated GnRH-I was added to compete with the preincubated peptides for binding to the antibodies.

This procedure was performed for serum collected before and after booster immunization, as specificity of the antibodies may increase after booster immunization.

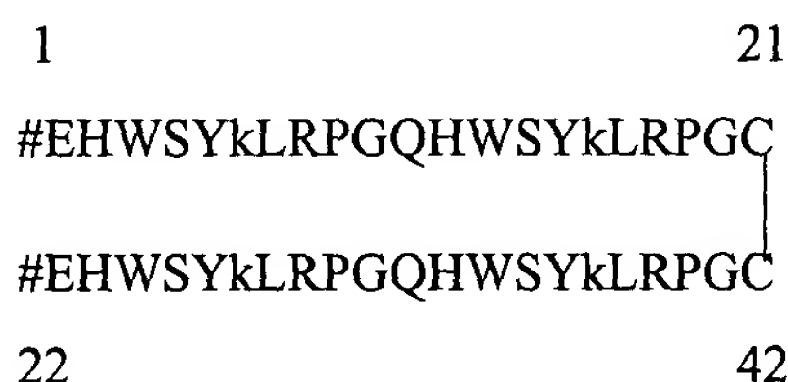
When the peptides according to the invention were used as the conjugate with ovalbumine (OVA-conjugate) and compared to controls, all showed effectiveness in the immunocastration of young male pigs. Comparison with the known G6k-GnRH tandem dimer OVA conjugate (see, Table 1), showed that the peptides according to the invention presented comparable or similar effectiveness even though their resemblance to the 'self'-hormone GnRH-I has decreased. The peptides according to the invention give an immunogenic response that allows for the effective discrimination between GnRH-I and GnRH-II. These peptides resulted in small testes and low testosterone levels. More specifically, the peptides expressing a low testes weight and a low testosterone level are R8A, G10A and S4A. The preferred peptides based on immunological selectivity between GnRH-I and GnRH II are S4A and pE1A.

In a preferred embodiment, the peptide is selected from the group consisting of pEHWA_YkLRPGQHWA_YkLRPGC# (SEQ ID NO: 5), pEHWSYkLAPGQHWSYkLAPGC# (SEQ ID NO: 6) and pEHWSYkLRPAQHWSYkLRPAC# (SEQ ID NO: 7). It is more preferably selected from the group consisting of pEHWSYkLAPGQHWSYkLAPGC# (SEQ ID NO: 6); and pEHWSYkLRPAQHWSYkLRPAC# (SEQ ID NO: 7).

In the peptides according to the invention, dimerisation of the tandem units can for example take place via the carboxyl-terminus or via the amino-terminus. Two

tandem units may for instance be dimerised by means of a disulfide or thioether bridge. To dimerise the tandem sequences the Cys at position 21 can be used, or Cys can be synthesized before the glutamic acid at position 1. Other methods to dimerise or multimerise the GnRH-tandem units can also be found in the prior art. If the Cys at position 21 is involved in the dimerisation and accordingly not available for coupling, it is likewise possible to use another amino acid of the tandem that can be coupled. If the dimerisation or multimerisation results in the loss of accessible sites where a carrier compound can be conjugated, it is sufficient to restrict the choice of replacement amino acids to an amino acid with an appropriate side chain. Such a replacing amino acid can be, for example, L or D-Lys, L or D-Glu or another amino acid containing a side chain that allows coupling to a carrier compound. Both L- and D- substitutions have been tested and found to have the same effect.

More in particular, an example of such a preferred peptide according to the invention, a D-Lys⁶-tandem-GnRH dimer (SEQ ID NO: 8) according to the following formula:



In this example of an embodiment of the invention it is possible to replace one of the amino acids of the tandem-dimer by another amino acid.

Other peptides or peptide sequences or coupled peptide sequences in which monomerised, dimerised or multimerised GnRH tandem units are present that contain amino acid substitutions are also part of the invention.

The invention further provides a composition which comprises a peptide brought into an immunogenic form. As a skilled worker knows, there are different methods of producing an immunogenic form of a substance which is in itself not immunogenic. One

possibility is to couple a peptide according to the invention to a suitable carrier protein. A suitable carrier protein is ovalbumin, KLH or BSA. In a tandem peptide, a cysteine at the - or C-terminus can be suitably used for a chemical coupling. In the tandem-dimer peptide, coupling can also be performed using the plain or the modified side chain of (D-) lysine, (D-) glutamine, or any other modified amino acid replacing amino acids of the peptide sequence. Suitable coupling methods and carrier proteins are well known to those of ordinary skill in the art.

According to the invention, there is preferred a composition which is characterized in that it comprises an immunogenic conjugate of a protein, such as ovalbumine, and a peptide or peptide composition.

A composition according to the invention can be used in the form of a vaccine. To this end the composition can be produced in a form that is suitable for administration. By administration of a vaccine according to the invention, an immunogenic response against GnRH is generated, preferably an immunogenic response against GnRH-I.

The invention therefore also provides a method of immunizing a mammal against GnRH-I, through vaccination of the mammal with a vaccine according to the invention. In a preferred embodiment, the invention provides a method of selectively immunizing a mammal against GnRH-I, with a vaccine according to the invention.

Of course, the vaccine preparation according to the invention can be combined with at least one immunoadjuvant. Suitable immunoadjuvants are known to those skilled in the art. A preferred adjuvant according to the invention can be Specol or a double oil emulsion, but other adjuvants that elicit no or only mild side-reactions can be used as well. The invention can be used in methods for immunizing individuals selected from a wide range of vertebrates, and particularly mammals, against GnRH-I. In a preferred embodiment of the invention, the vaccine can be administered in a single dose, which has the same efficacy as the presently known vaccines which must be administered in two-dose form. Immunization against GnRH-I, preferably selective, could for instance be used for the sterilization of, e.g., small domestic animals such as male and female cats and dogs, or for the treatment of aggressiveness in male dogs and bulls. Other conceivable reasons of immunization against GnRH-I with the present invention are

preventing heat in female animals such as dogs, cats and cows, and preventing or treating restlessness in male animals being fattened for slaughter. In human health care, immunization against GnRH, preferably selective against either GnRH-I or GnRH-II, can be used in the treatment of prostate cancer and breast cancer and, if required, in the treatment of some forms of pituitary carcinoma.

A preferred embodiment is a method of improving the carcass quality of pigs, wherein the pigs are vaccinated with such a vaccine preparation according the invention. The invention is illustrated in the following experimental part.

I. Immunocastration of pigs

MATERIALS AND METHODS

Materials

Acetonitrile (ACN) was HPLC-S gradient grade, N-methylpyrrolidone (NMP), diisopropylethylamine (DIEA) dimethylformamide (DMF), trifluoroacetic acid (TFA) and piperidine were peptide synthesis grade and were all obtained from Biosolve (Valkenswaard, NL). N-hydroxybenzotriazole (HOBt) and 2-(1H-benzotriazol-1-yl-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Richelieu Biotechnologies Inc. (Hamon, Canada). Benzotriazol-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBOP) was obtained from Novabiochem (Laufelfingen, Switzerland). Thioanisole (TA), ethanedithiol (EDT), dimethylsulfoxide (DMSO), pentane and dimethylaminopyridine (DMAP) were pro-analysis grade and were obtained from Merck (Darmstad, Germany). Diethylether was purified over a column of activated, basic aluminum oxide before use. Amino acid derivatives and resins were obtained from Bachem Feinchemicalien AG (Bubendorf, Switzerland).

Multiple peptide synthesis (MPS)

A Hamilton Microlab 2200 was programmed to deliver washing solvents and reagents to a rack with 40 individual 4 ml columns with filter, containing 30 mmol of resin for peptide synthesis. The columns were drained after each step by vacuum. The coupling cycle was based on Fmoc chemistry using double coupling steps:

1. NMP wash (1 ml)
2. 30% (v/v) piperidine/NMP (3 min, 0.5 ml)
3. 30% (v/v) piperidine/NMP (17 min, 0.5 ml)
4. NMP wash (5 x 1 ml)
5. double coupling (2 x 30 min)
6. NMP wash (2 x 1 ml)

Coupling step: Fmoc-amino acid in NMP (0.4 M, 0.25 ml), HBTU/HOBt (0.45 M, 0.22 ml) in DMF, and DIEA (2 M, 0.2 ml) in NMP were transferred to the reaction vessel and allowed to react for 30-50 min. The reaction mixture was drained and the coupling procedure was repeated once.

After coupling of the last amino acid, the Fmoc group was cleaved with 30% piperidine/NMP, the peptides were washed, acetylated in 30 min. using NMP/acetic anhydride/DIEA 10/1/0.2, washed again, and dried. The peptides were deprotected and cleaved in 2 hr in a mixture of 1.5 ml of TFA/phenol/TA/water/EDT 10/0.75/0.5/0.5/0.25 (reagent K). The cleavage mixture was filtered, the resin was washed with 0.5 ml TFA, and the peptide was precipitated by adding 13 ml pentane/diethylether 1/1. After centrifugation, the precipitate was extracted again with pentane/diethylether. The precipitate was dried, dissolved in ACN/water 1/1 and lyophilised. This procedure yields, depending on molecular weight, 25 to 70 mg of peptide.

Peptide sequences synthesized in single letter amino acid code are summarized in Table 1.

TABLE 1: Amino acid sequence in single letter code of peptides synthesized

peptide	amino acid sequence
G6k-GnRH-tandem	pEHWSYkLRPGQHWSYkLRPGC# (SEQ ID NO: 9)
pE1A-G6k-GnRH-tandem	* <u>A</u> HWSYkLRPG <u>A</u> HWSYkLRPGC# (SEQ ID NO: 10)
~ S4A-G6k-GnRH-tandem	pEHW <u>A</u> YkLRPGQHWS <u>A</u> YkLRPGC# (SEQ ID NO: 5)
Y5A-G6k-GnRH-tandem	pEHWS <u>A</u> kLRPGQHWS <u>A</u> kLRPGC# (SEQ ID NO: 11)
L7A-G6k-GnRH-tandem	pEHWSYk <u>A</u> RPQGHSYk <u>A</u> RPGC# (SEQ ID NO: 12)
~ R8A-G6k-GnRH-tandem	pEHWSYkL <u>A</u> PGQHWSYkL <u>A</u> PGC# (SEQ ID NO: 6)
30 P9A-G6k-GnRH-tandem	pEHWSYkLR <u>A</u> GQHWSYkLR <u>A</u> GC# (SEQ ID NO: 13)
~ G10A-G6k-GnRH-tandem	pEHWSYkLRP <u>A</u> QHWSYkLRP <u>A</u> C# (SEQ ID NO: 7)

pE = pyroglutamic acid; * = acetyl; # = amide; k = d-lysine; G6k = Gly on position 6 in the native GnRH sequence substituted by d-Lysine

Analytical HPLC

5 For analysis of peptides, we used a LC-MS (electrospray) system, which consists of two Waters pumps model 510, a Waters gradient controller model 680, a Waters WISP 712 autoinjector, and a Waters 991 photodiode array detector. The mass spectrometer was a Micromass Quattro II sq, which was used in positive ion mode. Products were analyzed in a linear gradient from 10% ACN/water with 0.05% TFA to 70% ACN/water
10 with 0.05% TFA in 30 min on a Waters Delta Pak C18-100Å (3.9x150 mm, 5 mm) column at 1 ml/min at 215 nm. All products were between 40-70 % pure according to the peak area.

Dimerisation

15 Crude products were dimerised by dissolving the products in 20% DMSO in water. The pH was adjusted to 5 - 6 with 1% NH_4HCO_3 , maintaining a clear solution. Correction of pH was done with 1% acetic acid. After stirring at room temperature for at least 5 h, the products were stored at -20°C until purification.

Preparative HPLC

20 Peptides purifications were carried out using a Waters Prep 4000 liquid chromatograph, equipped with a Waters RCM module with two PrepPak cartridges plus guard cartridge (40x210 mm or 25x210 mm) filled with delta-Pak C18-100Å (15 mm) material. In general, purifications were run using the same eluents as in analytical
25 HPLC, but at a gradient speed of 0.5% ACN/ min and a flow rate of 40 or 100 ml/ min. Peptides were detected at 215-230 nm using a Waters 486 spectrophotometer with a preparative cell. The peptides were lyophilised and purity was determined to be at least 90%.

30 Conjugate preparation

For conjugation via N-ethyl-N-(3'-dimethylaminopropyl)carbodiimide hydrochloride ("EDC") to chicken egg albumin ("OVA") an equal weight of both the peptide and the carrier protein were dissolved separately in milliQ water and both solutions were mixed well. Next a 10-fold excess, based on weight equivalents, of EDC was dissolved in milliQ water. Subsequently, this solution was slowly added to the solution of peptide/OVA under continuous stirring, pH of this final solution is 5. After at least 6 h slowly shaking the product was dialyzed (MW cut-off 10,000) against a 300 times excess of milli Q water for two days. Water was refreshed twice a day. The loading was calculated from comparative amino acid analysis of the conjugate and the carrier protein. Amino acid analysis was performed using a Waters Pico-Tag system, after hydrolysis in a Pico-Tag workstation using 6N HCl at 150 °C for 1 h, and derivatisation with phenyl isothiocyanate.

According to the amino acid analysis the conjugates contained between 0.3 and 0.5 mg of peptide per mg of carrier protein, with exception of the P9A-G6k-GnRH-tandem-dimer conjugate which only contained 0.16 mg peptide per mg ovalbumine.

The G6k-GnRH-tandem-dimer OVA conjugate is abbreviated by G6k-TD. The conjugates with alanine substitutions are abbreviated by the amino acid in the native GnRH sequence that is replaced, its position and an A for the Alanine replacement. For example pE1A-G6k-GnRH-tandem-dimer OVA conjugate is pE1A.

Emulsion preparation

Specol (Special Oil Phase, ID-DLO, Lelystad, The Netherlands) consisting of two detergents in a light mineral oil was used as oil phase (Bokhout et al., 1981). The water-in-oil-(WIO)-emulsions were prepared using an Ultra Turrax (Janke and Kunkel, Staufen, Germany) with a stirring bar i 25 mm. The oil phase Specol (5 parts v/v) was brought into a 25 ml glass vessel and the water phase (4 parts v/v) consisting the conjugate in milli Q water was slowly added while the emulsion was stirred. After the water phase was added, the emulsion was stirred for half a minute at the same rotation speed (15000 rpm). Emulsions were stored overnight at 4°C to check stability and were administered to the animals the next day.

Animals

Male piglets, approximately 10 weeks of age, were involved in this experiment. The crossbred piglets were housed in half slatted pens and were given ad libitum access to feed and water.

Immunization

The piglets were randomly assigned to the treatments, 6 or 7 piglets per treatment. All animals were injected with 2 ml emulsion containing the dimerised tandem GnRH conjugates (i.e. 62 mg peptide) or an emulsion without antigen. Injections were administered intramuscularly in the neck at the start of the experiment (day 0) and 7 weeks later (7wpv). Thirteen weeks after initial immunization (13 wpv) all animals were slaughtered.

Measurements and blood sampling

Animals were weighed at day 0 and 7 and 13 wpv. Testis sizes were determined by measuring testis length with a vernier calipers at day 0, and 7, 10 and 13 weeks thereafter. Testis sizes were recorded as average of both testicles.

Blood samples were taken via puncture of the vena jugularis on the same days testis sizes were measured, and also 4 weeks after the initial immunization. Blood samples were kept overnight at 4°C and the next day serum was obtained by centrifugation (1500 g, 15 min). Serum samples were stored at -20°C until assayed.

Evaluation after slaughter

After slaughter testes were removed, dissected free of epididymes and weighed. Testes weights were recorded as average of both testicles.

Peptide antibodies

Antibodies to the peptides used for immunization were determined with an ELISA. Peptides were coated in the wells of a microtitreplate using glutardialdehyde

(GDA). GDA was coated to the surface of the wells by incubation with 0.2% GDA in 0.1 M phosphate buffer (pH 5) for 4 hours at room temperature. Plates were rinsed 3 times for 10 minutes with 0.1 M phosphate buffer (pH 8). One microgram peptide in 100 ml phosphate buffer (0.1 M, pH 8) was coated per well by incubating for 3 hours at 37°C. Plates were stored at -20°C until used. Thawed plates were rinsed 3 times for 10 minutes with milli-Q water containing 8.2 g NaCl, 1.15 g Na₂HPO₄·2H₂O, 0.20 g NaH₂PO₄·2H₂O and 5 ml of a 10 % Tween 80 solution in water per litre water.

Serial serum dilutions of the anti-peptide sera were allowed to react with the coated peptides for 1 hour at 25°C. After rinsing for 3 times 10 minutes goat-anti-pig IgG coupled to horseradish peroxidase (Dako, Glostrup, Denmark) was introduced as second antibody for 1 hour and ABTS (Boehringer, Mannheim, Germany) ((250 ml (2 g/100 ml) in 10 ml substrate buffer to which 20 ml H₂O₂ (3% solution)) was used as substrate. Absorption was measured at 405 nm.

GnRH antibodies

Antibodies to GnRH were determined as described by Meloen et al. (Vaccine 12, 741-746 (1994)). Serial dilutions of the pig antisera were allowed to bind to ¹²⁵I-GnRH. Titers are expressed as percentage binding of ¹²⁵I-GnRH at a given serum dilution.

Testosterone

Testosterone levels in serum were determined using a Coat-a-Count kit purchased from DPC laboratories, Los Angeles, CA.

RESULTS

testis size and testis weight

Seven weeks after the first immunization, immunocastration effects already could be observed by measuring testicle sizes. Three treatments (R8A, G10A and G6k-TD) showed hardly any increment (<10mm) of average testis size at time of booster. These treatments were successful with testis weights at slaughter being 70 grams or less. Other treatments that were effective in terms of low testes weights are pE1A and S4A, while in

group Y5A, L7A and P9A, 2, 1 and 1 animals did not respond to the immunization respectively (Table 2). Individual testis weight of immunocastrated animals did not exceed 70 grams, resulting in a clear difference between immunocastrated and not immunocastrated animals.

5

TABLE 2: Effectiveness of the different treatments according testis weight (g)

	treatment	individual testis weight(g)	testis weight (g)	number
			(median (range))	responders/ total number
10	G6k-TD	10,10,10,12,16,70	11 (10-70)	6 / 6
	pE1A	16,18,33,36,53,55,65	36 (16-65)	7 / 7
	S4A	10,14,15,36,40,43,69	36 (10-69)	7 / 7
	Y5A	15,19,34,44,69,210,235	44 (15-235)	5 / 7
	L7A	12,13,15,20,24,29,195	20 (12-195)	6 / 7
	R8A	10,10,12,15,17,19,19	15 (10-19)	7 / 7
	P9A	8,9,20,23,31,57,300	23 (8-300)	6 / 7
15	G10A	11,13,14,14,15,30,39	14 (11-39)	7 / 7
	Controls	150,173,204,206,236	204 (150-236)	0 / 5

Antibody response

Mean antibody titers against the peptides used for immunization are given in table 3. Mean antibody titer of pigs treated with H2A and P9A are lower than peptide antibody titer of other treatments.

Antibody titers of individual animals against the different peptides ranged from 2 to 4. Within a treatment animals that were not immunocastrated showed the lowest anti-peptide titer. Animals treated with H2A and W3A were not immunocastrated, but significant anti-peptide titers were present.

GnRH antibody binding percentages at 1/ 2000 serum dilution were undetectable or low in H2A and W3A groups. However at a 1/200 serum dilution antibodies were detectable in sera of all animals of group H2A and three animals of group W3A.

Animals with low average GnRH antibody titers were not immunocastrated. High antibody titers did always result in successful castrated animals. Testicle weight of animals with intermediate antibody titers varied from 15 to 300 gram.

5 Average GnRH antibody titers per treatment showed a clear relation with testis weight (median) per treatment (Table 3).

Testosterone

10 Testosterone levels of all successful treated animals were low and decreased after second immunization. However a majority (n=31) of the animals reflected a castration effect as early as four weeks after initial immunization by showing undetectable testosterone levels. Testis weight of these animals varied from 8–36 grams.

The majority of the animals were thus effectively immunized after the administration of a single dose.

15 Although testosterone levels at slaughter were low for all immunocastrated animals, two animals with testes weights of 65 and 70 grams had significant serum testosterone levels of 3.80 and 1.18 pmol/ml respectively.

The peptides that resulted in low testes weight combined with undetectable testosterone levels are considered the most effective peptides for the immunocastration of pigs. These peptides are S4A, R8A and G10A.

20 Testosterone levels at slaughter of pigs that were not immunocastrated animals varied between 0.46 and 48.91 pmol/ml.

TABLE 3: Effect of different treatments on mean testis weight, peptide antibody titer, GnRH antibody binding percentage and LH and testosterone levels in serum.

25	Treatment	testis weight (median range)	anti peptide titer at 10wpv	GnRH binding (average)	testosterone (pmol/ml) at 13 wpv median (range)
	G6k-TD	11 (10-70)	3.32	17.2	n.d. (n.d.-1.18)
	pE1A	36 (16-65)	3.24	15.7	n.d. (n.d.-3.80)

5	S4A	36 (10-69)	3.19	16.9	n.d. (n.d.)
	Y5A	44 (15-235)	2.92	11.3	n.d. (n.d.-9.44)
	L7A	20 (12-195)	3.52	17.6	n.d. (n.d.-48.91)
	R8A	15 (10-19)	3.34	17.7	n.d. (n.d.)
	P9A	23 (8-300)	2.62	15.8	n.d. (n.d.-11.57)
	G10A	14 (11-39)	3.19	17.6	n.d. (n.d.)

wpv=weeks post vaccination, n.d.= not detectable

II. Replacement of D-amino acid with L-amino acid

10 D-lysine (k) at positions 6, 16, 27, and 37 in a tandem dimer is replaced by an L-lysine (K) and the resulting peptide tested (Table 4).

G6k-GnRH-tandem: pEHWSYkLRPGQHWSYkLRPGC# (SEQ ID NO: 9)

G6K-GnRH-tandem: pEHWSYKLRPGQHWSYKLRPGC# (SEQ ID NO: 14)

15 Table 4:

treatment	testis weight (g) median (range)	number responders/ total	anti peptide titer at 10 wpv	GnRH- binding (average)	testosterone in pmol/ml, at 13 wpv, (median)
G6k-TD	11 (10-70)	6/6	3.32	17.2	n.d.
G6K-TD	21 (9-175)	5/6	2.70	15.6	n.d.
Controls	204 (150-236)	0/5	n.d.	n.d.	2.05

20 n.d. : not detectable.

Replacement of D-lysine by L-lysine does not alter efficacy of the vaccine antigen.

25 III. Discrimination between GnRh-I and GnRH-II

An GnRH antibody binding competitive radioimmunoassay was performed in order to determine whether antibodies raised against G6k-GnRH-tandem (SEQ ID NO: 9) dimer peptide or its alanine replacement analogs bind to GnRH-II or lack binding to GnRH-II. Serum dilutions were preincubated with either GnRH-I, GnRH-II, a control

peptide or no peptide. Next iodinated GnRH-I was added to compete with the preincubated peptides for binding to the antibodies.

This procedure was performed for serum collected before and after booster immunization, as specificity of the antibodies may increase after booster immunization due to antibody maturation.

Materials and methods

Serum samples of the 10 wpv bleeding (3 weeks post booster immunization) were diluted 1/10000 in PBS with 0.4 % BSA (dilution buffer). Fifty μ l serum dilution was put in microwell plates and 25 μ l peptide solution (0, 0.25, 2.5 or 25 pmol peptide in dilution buffer per well) was added. This mixture was allowed to incubate for 24 hours at 4°C. The next day 25 μ l iodinated GnRH (approximately 13000 cpm) was added and after overnight storage (4°C) unbound peptide was separated from bound peptide with charcoal. After centrifugation supernatant was separated, counted and the percentage iodinated GnRH bound to the antibodies was calculated.

Antibodies collected after the first immunization (7 wpv) were tested in a 1/2000 serum dilution and only one peptide dilution was tested (25 pmol/well).

Results

Binding of iodinated GnRH at a 1/10000 serum dilution was observed for the sera of all treatments except for H2A and W3A sera. Competition with GnRH-I resulted in a dramatic reduction of binding of iodinated GnRH to the antiserum (see Figure 1). At the sera dilution we tested, inhibition of binding of iodinated GnRH by GnRH-I was dose dependent for most sera. As expected, none of the antisera bound to the control peptide.

Competition by GnRH-II for antibody binding to iodinated GnRH resulted in a reduced binding in all sera of R8A and G10A treatments, in most of the sera of treatments P9A and TDK and in half of the sera of treatments Y5A and L7A (see Figure 2). However, antibodies of substantially all animals of the pE1A and the S4A group completely lacked binding to GnRH II, and were thus specific for GnRH I.

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